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Clinical Significance of Retinoblastoma Protein (pRB) Expression in Esophageal Squamous Cell Carcinoma

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Background and Objectives: The goal was to evaluate the clinicopathological significance of retinoblastoma gene product (pRB) expression in esophageal squamous cell carcinoma.
Methods: We investigated abnormal pRB expression in tumors in 191 patients using an immunohistochemical method in conjunction with anti-RB protein antibody. Surgically resected esophageal squamous cell carcinomas were examined by immunohistochemical analysis for altered pRB expression.
Results: Decreased pRB nuclear staining indicating loss of RB function occurred in 82 (43%) of the cases studied. The incidence of decreased pRB expression was higher in tumors with invasion to the adventitia (50%) than in tumors without invasion to the adventitia (33%, $P = 0.0188$). In addition, the incidence of decreased pRB expression was higher in tumors with lymph node metastasis (50%) than in those without (34%, $P = 0.0346$). The 3-year survival rates of 82 patients who had tumors with decreased pRB expression (30%) was significantly lower than that of 109 patients who had tumors with normal pRB expression (52%, $P = 0.0032$). However, in the multivariate survival analysis, pRB expression was not an independent prognostic factor for patients with esophageal squamous cell carcinoma.
Conclusions: Abnormal pRB expression appears to be closely associated with tumor development. However, the existence of tumors with hyperphosphorylated RB protein (inactivated form) in pRB-positive tumors, such as those in the present study, should be considered. Thus, discrimination of this hyperphosphorylated form of RB protein from the unphosphorylated RB protein is needed.
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KEY WORDS: **immunohistochemical staining; depth of tumor invasion; lymph node metastasis; prognosis**

INTRODUCTION

Uncontrolled G1-S transition in the cell cycle leads to abnormal cell proliferation and tumor development. The retinoblastoma gene was the first tumor-suppressor gene to be identified. The protein product of the retinoblastoma gene (pRB) plays an important role in regulating the ability of cells to enter the S phase of the cell cycle, the period during which DNA is synthesized. The cell cycle is controlled by phosphorylation/dephosphorylation of pRB [1].

The potential prognostic significance of altered pRB expression (absence or reduction of pRB expression) in various tumors has been the subject of several recent studies [2–6]. Xu et al. [7] reported that mutations of the retinoblastoma gene corresponded to loss of pRB expression and the intact gene expressed nuclear pRB. In

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TABLE I. Relationship Between pRB Expression and Clinicopathological Findings of 191 Patients With Surgically Resected Esophageal Squamous Cell Carcinoma

Variable	pRB-negative (n = 82)	pRB-positive (n = 109)	P
Age (years, mean ± SD)	62.1 ± 9.5	64.2 ± 9	0.1595
Gender (male/female)	74/8	97/12	0.7795
Histologic type of tumor			
Well differentiated	7	7	
Moderately differentiated	39	52	0.8496
Poorly differentiated	36	50	
Maximum diameter of tumor (cm, mean ± SD)	5.6 ± 2.7	5.5 ± 2.8	0.8283
Depth of tumor invasion (without invasion to the adventitia/with invasion to the adventitia)	26/56	53/56	0.0188
Lymph node metastasis (absent/present)	31/51	58/51	0.0346
Lymphatic invasion (absent/present)	30/52	58/51	0.0225
Blood vessel invasion (absent/present)	65/17	88/21	0.8017
Histologic stage			
0	0	3	
I	10	25	
IIA	18	22	0.0677
IIB	6	15	
III	41	37	
IV	7	7	

SD = standard deviation. Stage 0: Tis, N0, M0.

esophageal squamous cell carcinoma (SCC), frequent loss of heterozygosity at the retinoblastoma locus has been reported [8,9]. However, the prognostic importance of pRB expression in esophageal SCC remains unclear. Immunohistochemical analyses of RB gene product expression using an anti-RB protein antibody have recently been performed in some human malignant neoplastic tissues [2–7]. To evaluate the clinicopathological significance of pRB expression in esophageal SCC, we investigated abnormal pRB expression in tumors using an immunohistochemical method in conjunction with anti-RB protein antibody.

MATERIALS AND METHODS

Patients and Tumor Specimens

Between 1981 and 1998, 191 patients with primary SCC of the esophagus underwent esophagectomy at our hospital. To evaluate the prognostic significance of pRB expression in esophageal SCC, we investigated 191 tumors and 191 normal esophageal epitheliums adjacent to tumors from the patients included in the study (171 males, 20 females; age range: 39 to 84 years, mean: 63.3 years, median: 65 years). Usually, normal samples were obtained from the proximal resection margins. However, if the distance between the proximal resection margin and the proximal edge of tumor was less than 5 cm, normal epithelium was obtained from the distal side of the esophagus. The distance between normal sample and tumor was approximately 10 cm. None of the patients had received chemotherapy or radiation therapy preoperatively. All were subjected to total or subtotal esophagectomy. Esophagectomy was performed by right thoracotomy and laparotomy with mediastinal and abdominal lymphadenectomy. Reconstruction was done by esophago-gastrostomy using a gastric tube through the retrosternal route.

Histopathological Examination

The resected specimens were fixed in 10% buffered formalin for 24 hr. After fixation, all specimens were cut into 5-mm slices. The slices were embedded in paraffin, and tissue paraffin blocks (5 mm × 30 mm × 20 mm) were cut into 4-μm-thick sections for hematoxylin-eosin and immunostaining. The histopathological diagnoses were defined according to the guidelines for TNM classification [10]. All 191 tumors were diagnosed as squamous cell carcinoma (SCC); 14 tumors were diagnosed as well-differentiated SCC, 91 tumors were diagnosed as moderately differentiated SCC, and 86 tumors were diagnosed as poorly differentiated SCC. The pathological stages of 191 esophageal SCC were as follows: stage 0, 3 (tumors existed only in epithelial layer and did not invade to lamina propria); stage I, 35; stage IIA, 40; stage IIB, 21; stage III, 78; and stage IV, 14 (3 patients had liver metastasis and 11 patients had lymph node metastases around the coeliac or hepatic arteries at the time of operation).

Immunohistochemical Detection of RB Protein

The paraffin-embedded sections from tumors and normal esophageal epitheliums adjacent to tumors were stained by a specific mouse monoclonal antibody raised against human pRB (clone Rb1, diluted 1:40; DAKO, Glostrup, Denmark). Briefly, 4-μm-thick sections were dewaxed using xylene and transferred to alcohol. The slides were then placed in citric acid buffer (10 mM) and heated in a microwave oven (700 W) for 12 min to expose antigens. Endogenous peroxidase activity was blocked by incubating sections with 0.3% hydrogen peroxide in methanol for 30 min. Slides were then washed 3 times in phosphate-buffered saline (PBS) and incubated in 10% normal goat serum for 20 min to reduce nonspecific antibody binding. After washing with PBS, slides were incubated overnight at 4°C with the primary antibody. Nonspecific mouse IgG1 was used as a negative control. After washing the slides with PBS, biotinylated antibodies against mouse immunoglobulin were applied as second antibodies (Histofine ABC Kit, Nichirei, Tokyo, Japan) for 30 min. The reaction products were visualized with diaminobenzidine as the chromogen and the slides were counterstained with methyl green.

In all cases, adequate nuclear staining was obtained in the normal esophageal epitheliums, which represented a positive control. For the pRB immunostaining, only nuclear staining was considered as evidence of functional pRB. Immunostaining results were assessed, taking into account the pRB-positive cancer cells in the tumors. If $\geq$ 50% of the cancer cells were stained with pRB, tumors were determined to be pRB-positive. When < 50% of the cancer cells were stained, tumors were determined to be pRB-negative. pRB expression was determined by 2 independent observers (SO and YG) who had no knowledge of the patients' histories.

Statistical Analysis

The χ^2 test and Fisher exact probability test were used to compare frequencies. The differences in the numerical data between the 2 groups were evaluated by the Mann-Whitney U test. Patients were followed up for a median time of 12 months (range: 2 months to 175 months). Survival rates were calculated by the Kaplan-Meier method. Corrected survival rates were used; that is, only deaths caused by esophageal cancer were taken as outcome events and all other deaths were considered censored events. The log rank test was used to compare the survival curves of patients with pRB-positive and of patients with pRB-negative tumors. The influence of each variable on survival was assessed by the Cox proportional-hazard model. *P* values < 0.05 were considered to be statistically significant.

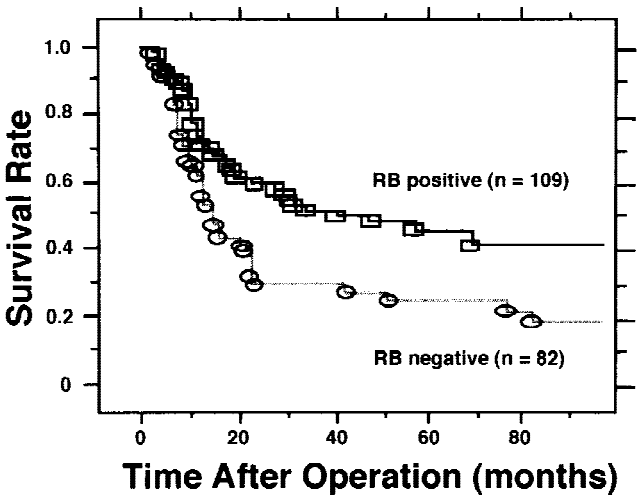

Fig. 1. The 3- and 5-year survival rates of 82 patients with pRB-negative tumors were 30% and 25%. The 3- and 5-year survival rates of 109 patients with pRB-positive tumors were 52% and 46%. The survival curve of patients with pRB-positive tumors was significantly higher than that of patients with pRB-negative tumors, as determined by the log rank test ($P = 0.0032$).

RESULTS

In 191 patients who underwent esophagectomy, no postoperative complications were detected in 152 patients. Thirty-nine patients had postoperative complications (operative morbidity rate was 20.4%), of which 19 died from postoperative complications within 30 days (30-day operative mortality rate was 9.9%) and 10 died within 60 days. The in-hospital mortality rate was 15.2% (29/191). Anastomotic leakage was detected in 22 patients (8 patients had pyothorax), 12 patients had pneumonia, 3 patients had necrosis of gastric tubes, and 2 patients had intrathoracic hemorrhage.

Eighty-two (43%) of the 191 esophageal SCCs analyzed showed negative pRB expression. pRB status was analyzed in relation to various clinical and clinicopathological characteristics (Table I). The percentage of tumors with invasion to the adventitia, that of tumors with lymphatic invasion and that of tumors with lymph node metastasis, were significantly higher in the 82 pRB-negative tumors than in the 109 pRB-positive tumors. However, there were no significant differences in age, gender, histologic types of tumors, or blood vessel invasion between pRB-positive and pRB-negative tumors (Table I).

The overall 3- and 5-year survival rates of 191 patients with esophageal SCC were 43% and 37%, respectively. The 3- and 5-year survival rates of 82 patients with pRB-negative tumors (30% and 25%) were significantly lower than those of 109 patients with pRB-positive tumors (52% and 46%, $P = 0.0032$; Fig. 1). Five parameters affected the survival rates of patients and the 3- and 5-year survival rates are as follows: 1. maximum diam-

TABLE II. Risk Factors Affecting Overall Survival Analyzed by Cox Proportional-Hazard Model

Variable	Hazard ratio	95% CI	*P*
Tumor size >5 cm vs. ≤5 cm	1.479	0.9–2.43	0.1228
Depth of tumor invasion (with invasion to the adventitia vs. without invasion to the adventitia)	2.451	1.351–4.444	0.0031
Lymph node metastasis (present vs. absent)	3.049	1.642–5.65	0.0004
Lymphatic invasion (present vs. absent)	1.248	0.725–2.151	0.4243
Blood vessel invasion (present vs. absent)	1.541	0.953–2.488	0.0775
pRB expression (negative vs. positive)	1.214	0.781–1.885	0.3884

CI = confidence interval. The 191 patients were divided into 2 subgroups according to the median size of their tumors (5 cm).

eter of tumor (≤ 5 cm: n = 103, 62% and 58%; > 5 cm: n = 88, 25% and 19%; $P < 0.0001$; the cutoff of the 2 groups was based on the median extent of the maximum diameters of 191 tumors); 2. depth of tumor invasion (without invasion to the adventitia: n = 79, 71% and 68%; with invasion to the adventitia: n = 112, 25% and 18%; $P < 0.0001$); 3. lymph node metastasis (absent: n = 89, 73% and 70%; present: n = 102, 23% and 15%; $P < 0.0001$); 4. lymphatic invasion (absent: n = 88, 67% and 62%; present: n = 103, 26% and 20%; $P < 0.0001$); and 5. blood vessel invasion (absent: n = 153, 51% and 45%; present: n = 38, 18% and 14%; $P = 0.0012$). Multivariate survival analysis was performed using Cox proportional-hazard model among these prognostic factors in patients with esophageal SCC. Only the depth of tumor invasion and the lymph node metastasis were detected as independent prognostic factors among the variables (Table II).

Mean disease-free survival time of surviving 162 patients (29 patients who died from postoperative complications were excluded) was 27.4 months (range: 1 month to 175 months). Mean disease-free survival time of 96 patients who had pRB-positive tumors was 30 months, significantly longer than that of 66 patients who had pRB-negative tumors (23.8 months, $P = 0.0065$).

DISCUSSION

The retinoblastoma gene product acts to control cell proliferation through regulation of the cell cycle at the G1-S-phase transition. The RB protein is bound to transcription factor E2F during the G1 phase. However, when pRB is phosphorylated by the cyclin-dependent kinase complexes, E2F is released and the cell can initiate DNA synthesis [11,12]. Cyclin-dependent kinase complexes accomplish phosphorylation of pRB, and these complexes are inhibited by p21, p27, and p16, the so-called cyclin-dependent kinase inhibitors [13,14]. Thus, abnormalities of upstream regulators of pRB phosphorylation might result in pRB hyperphosphorylation and inactivation. Abnormalities at the RB gene locus such as mutation and/or deletions and inactivation of expression may cause the reduced expression of pRB. These factors may negatively affect cell control.

In several types of tumors, reduced expression of pRB has been reported as a indicator of poor prognosis [4,15–17]. Granax et al. [18] suggested that the retinoblastoma protein family controls cell-cycle progression, cell differentiation, and apoptosis. In addition, Nakamura et al. [4] reported that in malignant astrocytoma with reduced expression of pRB, there was increased proliferative activity and a low apoptotic index. These observations suggest that pRB abnormalities may play an important role in tumorigenesis and tumor progression.

In esophageal SCC, the loss of heterozygosity at the *RB* locus was reported as 54% [8]. This result indicates that a considerable number of tumors might show reduced expression of pRB in esophageal SCC. However, the clinicopathological significance of pRB expression in esophageal SCC remains unclear. In the present study, the loss of pRB expression was detected in 43% of the esophageal SCCs that were analyzed. The percentage of stage III and IV in pRB-negative tumors (59%) was significantly higher than that of stage III and IV in pRB-positive tumors (40%, $\chi^2 = 6.188$, $P = 0.0129$). The survival curve of patients with pRB-negative tumors was significantly lower than that of patients with pRB-positive tumors. These results indicate that reduced pRB expression in tumors strongly correlates with tumor progression in esophageal SCC.

However, pRB expression was not an independent prognostic factor for patients with esophageal SCC. Similar results were obtained in studies of non-small cell lung cancer [3,16]. In the present study, we analyzed pRB abnormality by using immunohistochemistry, which is considered to be a sensitive and reliable method for detecting RB gene abnormality [19]. Xu et al. [16] have reported that the majority of known *RB* gene mutations result in a total loss of RB protein expression. Even when altered pRB is expressed in a rare tumor, it usually does not enter the nucleus and consequently does not produce false-positive nuclear staining. However, Cote et al. [15] reported that in cases of bladder cancer, patients with

high levels of pRB nuclear reactivity had an essentially identical rate of recurrence and the survival of such patients was as low as that of patients with tumors that did not express pRB. Moreover, Martinez et al. [20] reported a significantly positive correlation between Ki-67 growth fraction and pRB expression in pRB-positive lymphoma. These results strongly suggest that pRB expression detected by immunohistochemistry might show not only unphosphorylated RB protein (activated form) but also hyperphosphorylated RB protein (inactivated form). Thus, in the present cases, some pRB-positive tumors might have hyperphosphorylated RB protein (inactivated form); the prognosis of patients with such tumors would be as poor as that of patients with pRB-negative tumors. The development of a method of discriminating hyperphosphorylated RB protein from unphosphorylated RB protein will help explain the prognostic significance of pRB in esophageal SCC.

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